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Award Number: **W81XWH-12-1-0258**

TITLE: Neuroprotection and Anti-Epileptogenesis with Mitochondria-Targeted Antioxidant

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REPORT DATE: October 2014

TYPE OF REPORT: Annual Project Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual Progress Report	30 Sep 2013 - 29 Sep 2014

4. TITLE AND SUBTITLE
Neuroprotection and Anti-Epileptogenesis with Mitochondria-Targeted Antioxidant

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0258

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) Jeffrey H. Goodman

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Research Foundation For Mental Hygiene
Institute for Basic Research
1050 Forest Hill Road
Staten Island, New York 10314

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT The goals of the project were to assess the neuroprotective and antiepiletogenic properties of a mitochondrial-targeted antioxidant, SS-31 in the pilocarpine (PILO) model of status epilepticus (SE), the kindling seizure model and the tetanus toxin (Tx) model. Progress on the project was limited during the initial grant period due to an inability to obtain a sufficient quantity of SS-31 to perform the proposed experiments. As a result a no cost extension was granted in April 2014. More SS-31 was obtained from Stealth Peptides, Inc. on May 29, 2014. The experiments performed during initial grant period focused on Aim#1. In these experiments adult male Sprague-Dawley rats (260-405g) were pretreated with SS-31 (3 or 10mg/kg, sc; n=14) or saline (n=10) 45min before induction of SE with Pilo (365mg/kg, sc). The results suggested that SS-31 had no effect on the latency to SE and that there was no evidence of neuroprotection in hippocampal tissue stained for: Nissl, Fluoro-jade C (FJ), NeuN and heat shock protein (HSP). The insult generated by prolonged seizure activity appeared to be too severe for SS-31 to be effective. For the current reporting period we extended the experiments in Aim#1 and initiated experiments in Aim#2 testing the efficacy of SS-31 in the kindling seizure model. We addressed the possibility that prolonged seizure activity was too severe an insult for SS-31 to be effective by testing SS-31 (10mg/kg, sc) in PILO-treated rats (6 controls; 6 experimentals) where the duration of SE was limited to 5min and the dose of diazepam used to attenuate SE was increased from 5mg/kg, ip to 10mg/kg, ip. The brains from these animals have been sectioned and staining for the markers listed above has been initiated. To date the only marker that has been evaluated is FJ which revealed that despite limiting the duration of SE, SS-31 was not neuroprotective. We are in the process of completing the remaining stains. For Aim#2, a kindling stimulus was delivered to the hippocampus of each rat and the stimulus-induced afterdischarge (AD) threshold and duration were determined. A minimum of 24hr later SS-31 (10-20mg/kg, sc; n=9) was administered 30min before AD testing. SS-31 had no effect on AD threshold but there was evidence that SS-31 (20mg/kg) decreased AD duration. These experiments need to be repeated.

15. SUBJECT TERMS
Neuroprotection, antiepileptogenesis, antioxidant

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	9	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION:
A number of studies have provided evidence that reactive oxygen species play a role in the induction of seizures and seizure-induced neuronal death. The goals of this project are to test the efficacy of a novel, mitochondrial-targeted antioxidant SS-31, as a neuroprotective and antiepileptogenic agent in three experimental models of epilepsy. The pilocarpine-induced model of status epilepticus (PILO) will be used to test SS-31 as a neuroprotectant, the kindling model will be used to test SS-31 as an antiepileptogenic and anticonvulsant agent, and the tetanus toxin model (TX) will be used to test SS-31 as an anticonvulsant. If SS-31 proves to be effective in these studies future experiments will test SS-31 in models of traumatic brain injury.

Body:
SS-31 was created by Dr. Szeto but the rights to the drug are controlled by Stealth Peptides, Inc. Progress on the project during the initial grant period was limited due to limited access to the test agent, SS-31.This lead to the granting of a no-cost extension in April of 2014. At the end of May 2014 the PI was able to acquire a new supply of SS-31. Therefore the drug was available for testing for only 5 months of the current reporting period. In the current grant period we have expanded our preliminary studies using the PILO model (Aim #1), and began testing SS-31 in the kindling model (Aim#2).

Aim #1 – Test the neuroprotective and anticonvulsant properties of SS-31 in the pilocarpine model of status epilepticus (SE) in the rat.
In this model, prolonged seizure activity causes neuronal cell death in specific neuronal populations in the rodent hippocampus. Adult male Sprague-Dawley rats (260-405g) were used. In our initial experiments, experimental animals were pretreated with SS-31 (3 or 10mg/kg, sc) 45min before induction of SE with pilocarpine (365mg/kg, sc). One hour after the onset of SE each animal received an injection of diazepam (5mg/kg, ip) to attenuate SE and to improve survival. Control animals received an injection of saline instead of SS-31. To determine whether treatment with SS-31 affected the development of SE, the time to the onset of SE was measured for control and SS-31 treated animals. To examine neuroprotection the animals were perfusion-fixed with 4% paraformaldehyde (PAF) 1-3 days after SE. The brains were sectioned on a vibratome through the dorsal hippocampus. Sections were processed for the following histochemical and immunohistochemical stains: Nissl, Fluoro-jade C (FJ), NeuN and heat shock protein 70-72 (HSP). Nissl, FJ and NeuN stains were used to assess neuroprotection. HSP was used to detect neuronal stress but under some conditions has been shown to be neuroprotective.

Preliminary results from the initial grant period 1) there was no significant difference in the latency to the onset of SE between control and SS-31 treated rats 2) The was no evidence of neuroprotection in any of the stains examined. We did observe an increase in the expression of HSP in the granule cells of SS-31-treated rats. The failure of SS-31 to delay the onset of SE and to protect vulnerable neurons suggests that it is ineffective in this model. The insult induced by the prolonged seizure activity associated with SE may be too great for SS-31 to be effective. However, the observation that SS-31 increased HSP staining in the dentate granule cells suggests that SS-31 had an effect on the network.

Results from Current Grant Period - Aim #1
1) We repeated the above study injecting SS-31 (10mg//kg, sc, n=4) 45min before induction of SE with PILO (365mg/kg, sc). The animals were perfusion-fixed with 4% PAF, and the brains were removed, sectioned and stained as described above. Stained tissue from SS-31 treated rats was compared to tissue from 2 saline-treated controls. Similar results were obtained in this experiment to what we had previously reported. SS-31 was not neuroprotective and did not delay the onset of SE.

2) To pursue the possibility that PILO-induced SE was too severe an insult to observe a neuroprotective effect of SS-31 we tested SS-31 in a modified version of the PILO model. To limit the amount of PILO-induced seizure activity we administered a higher dose of diazepam (10mg/kg, ip) 5min after the onset of SE instead of the usual 60min. We also injected SS-31 into the experimental rats 30min before the

injection of PILO instead of 45min. We hoped these modifications would sufficiently limit the amount of seizure activity and make it easier to detect a neuroprotective action of SS-31. Using this modification of the PILO model, SE was induced in 6 control and 6 experimental rats. Three days after SE the animals were perfusion-fixed with 4% PAF, the brains removed and sectioned (50µm) on a vibratome. Slide-mounted sections were stained for Nissl and FJ and free floating sections will be immunostained for: NeuN, HSP and GFAP a marker of astrocytes. We are still in the process of completing the inmmunohistochemical staining of this tissue. To date the only stain evaluated was FJ. There was no difference in FJ staining in SS-31-treated tissue compared to controls suggesting that even with reduced seizure activity there was no evidence of neuroprotection with SS-31. Once the other stains are completed and the tissue evaluated we will definitively determine whether SS-31 has any neuroprotective efficacy in this model.

Aim #2 –Test the antiepileptogenic properties of SS-31 in the kindling seizure model. Kindling is a seizure model where repeated, spaced delivery of an initially subconvulsive stimulus to a limbic structure results in a permanent change in brain function such that eventually the kindling stimulus regularly elicits a limbic seizure. Progression through the kindling process can be assessed by measurement of the severity of the behavioral seizure and by measurement of the threshold and duration of the electrographic afterdischarge (AD). Behavioral seizures are scored on a 1-5 scale with stages 1-2 being equivalent to partial seizures and stages 3-5 equivalent to generalized convulsions. Once animals have exhibited 3 consecutive stage 5 seizures the kindling process is considered complete the animals are considered to be fully kindled. The number of stimulations required to reach a given stage, AD threshold and AD duration can be measured to assess the epileptogenic process. AD threshold is determined by delivering a kindling stimulus at a low current intensity. If no AD is detected the delivery of the kindling stimulus is repeated at a higher current intensity until an AD is elicited. AD testing is initiated at a current intensity of 5µA and increased in 5µA increments until an AD with a duration of at least 5sec was observed.

Effect of SS-31 on AD threshold and duration

The purpose of this first experiment was to determine if pretreatment with SS-31 altered AD threshold and AD duration. Each of the rats acted as their own control. Bipolar platinum depth electrodes were stereotaxically implanted bilaterally into the dorsal hippocampi of 13 anesthetized adult male Sprague-Dawley rats. Several screws electrodes were also implanted into the skull to allow for recording of surface EEG and to act as a ground. The electrodes were connected to a headstage which allows for connection to a stimulation/EEG recording system through a cable. Animals were allowed to recover from electrode implantation surgery a minimum of one week before entering into the study. The kindling stimulus is delivered through the same electrodes used to record EEG activity in an awake, freely-moving animal. Data were collected from 9/13 rats as 4 rats were removed from the study due to poor recording quality or loss of the electrode head stage. The experimental design consisted of initially determining baseline AD threshold and AD duration. A minimum of 24hr after the determination of baseline AD threshold and duration each animal was treated with SS-31, 30min before delivery of the kindling stimulus. This pattern was continued over a number of days. For this experiment the kindling stimulus had the following characteristics: 60Hz, 1msec biphasic pulse, delivered for 2sec.

Results:

We initially tested SS-31 at dose of 10mg/kg, sc., in 5 rats. Figure 1 illustrates that pretreatment with SS-31 at a dose of 10mg/kg had no effect on AD threshold (Figure 1A) or AD duration (Figure 1B). However, when the dose of SS-31 was increased to 20mg/kg (n=9), SS-31 still had no effect on AD threshold (Figure 2A) but **decreased AD duration in 6/9 rats** (Figure 2B). Figure 3 illustrates an electrographic example of a shortened AD after treatment with SS-31. Since during the normal kindling process the AD duration increases with continued daily stimulation these data suggest that SS-31 (20mg/kg) shorted the duration of the stimulation-induced epileptiform activity. It also suggests that repeated administration of SS-31 throughout the kindling process could interfere with epileptogenic process such that the development of generalized seizures could be delayed or prevented. This current experiment will need to be repeated to confirm these results. We will then test SS-31 during kindling acquisition to determine whether it actually delays the epileptogenic process. The observation that we did not see an effect of SS-

31 at doses less than 20mg/kg raises the possibility that SS-31 is not crossing the blood brain barrier as readily as we had anticipated. To address this issue we also plan on testing SS-31 in this model by delivering it directly into the hippocampus through and in dwelling cannula to see if that will improve its efficacy.

Aim #3 – Test the anticonvulsant properties of SS-31 in the Tetanus Toxin (TX) model of mesial temporal lobe epilepsy. The TX model is established and run on a regular basis in the laboratory. TX when injected into the hippocampus induces spontaneous seizures without causing a lesion. We will initiate and complete these experiments during the remaining period of the grant.

KEY RESEARCH ACCOMPLISHMENTS:

- We were able to obtain a sufficient quantity of SS-31 to complete the proposed experiments
- Treatment with SS-31 did not delay the onset of status epilepticus in the pilocarpine model
- SS-31 was not neuroprotective in the pilocarpine model of status epilepticus
- Treatment with SS-31 altered the expression of HSP in the granule cells of the dentate gyrus after status epilepticus however the significance of this observation is unclear.
- SS-31 at a dose of 20mg/kg shortened AD duration after a kindling stimulus delivered to the dorsal hippocampus in 6/9 animals. This experiment needs to be repeated to confirm these results but the results suggest that SS-31 could interfere with the epileptogenic process.

REPORTABLE OUTCOMES:
None at this time.

CONCLUSIONS:
Progress on the project has been limited due to limited access to the test compound. This resulted in the grant requiring a no-cost extension since a sufficient supply of the test compound was not received before the end of the original grant period. At the end of May, 2014 additional drug was received. This allowed for continued progress on Aim #1 and initiation of experiments outlined in Aim #2. Experiments in Aim#1 focused on the an examination of the neuroprotective properties of SS-31 and whether treatment with SS-31 could delay the onset of SE. In both cases the data were negative suggesting that the prolonged seizure activity associated with SE was too severe for SS-31 to have an effect. In the current grant period we repeated our initial experiments but also modified the PILO model in an attempt to decrease the amount of seizure activity to increase the possibility that a neuroprotective action of SS-31 could be detected. The data obtained so far in the current grant period confirmed our initial observations; however several immunostains still need to be evaluated from tissue generated using the modified PILO model. In our initial experiments treatment with SS-31 did alter the expression of HSP in the granule cells of the dentate gyrus. HSP is a marker of neuronal stress but has also been shown to be neuroprotective. This change in HSP expression suggests that SS-31 is interacting with the network but it unclear whether the increase in the granule cells is positive or negative. For Aim #2 we performed experiments addressing the efficacy of SS-31 in the kindling model by assessing changes in AD threshold and AD duration. SS-31 at a dose of 10mg/kg had no effect on AD threshold or duration. At a dose of 20mg/kg we observed a decrease in AD duration but no effect on threshold. These data suggest that SS-31 had no effect on seizure initiation but shortened seizure duration which potentially could delay or prevent the epileptogenic process. These experiments will need to be repeated before SS-31 can be tested in an kindling acquisition study.

References: None

APPENDICES: None

SUPPORTING DATA:

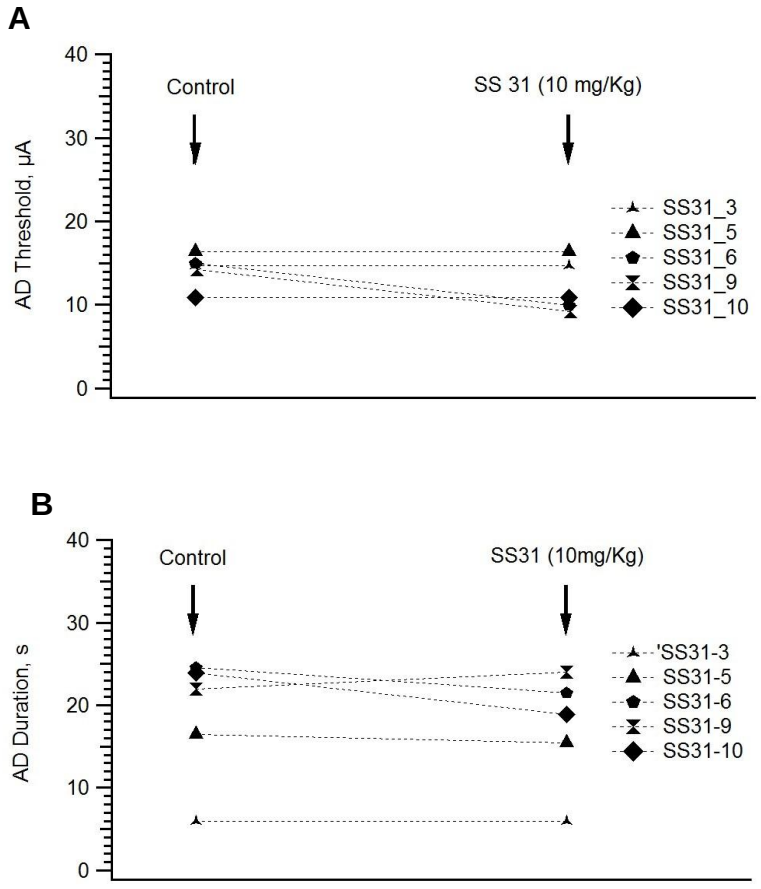

Figure 1 – Effect of SS-31 (10mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold or duration (n=5).

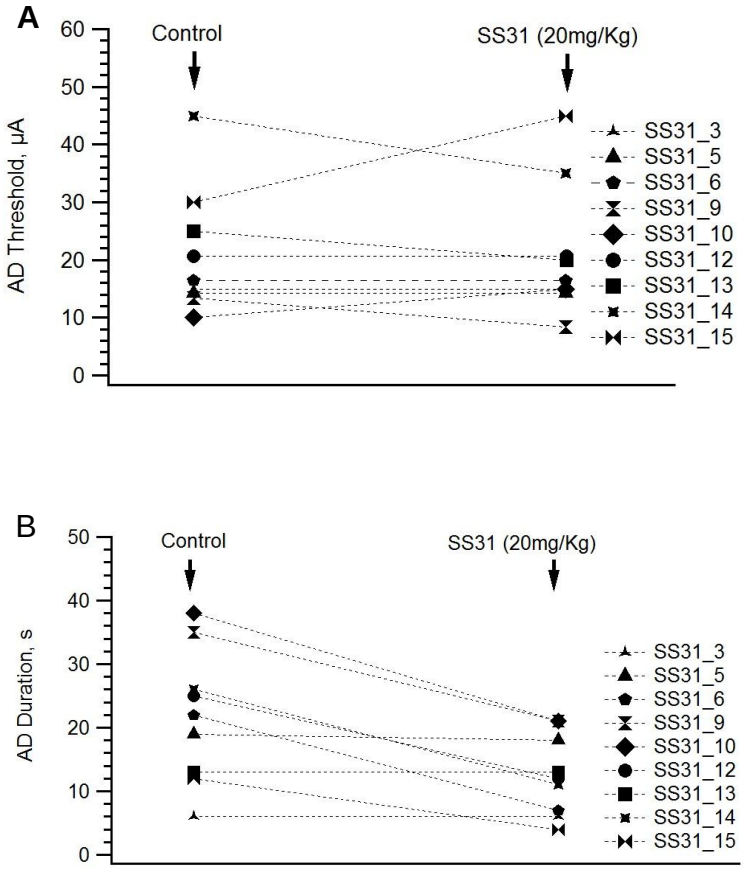

Figure 2 – Effect of SS-31 (20mg/kg, sc) on kindling AD threshold (A) and duration (B). SS-31 injected 30min before delivery of the kindling stimulus had no effect on AD threshold but decreased AD duration in 6/9 animals (n=9).

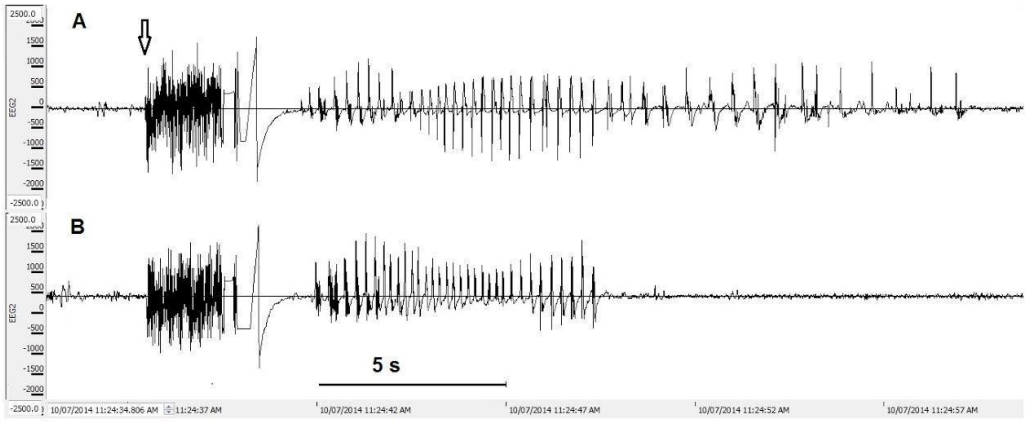

Figure 3 – Electrographic illustration of SS-31-induced decrease in kindled AD duration from the dorsal hippocampus of the rat. AD induced by a 20µA kindling stimulus. A. Baseline AD. B. AD 30min after SS-31 (20mg/kg, sc), Arrow indicates onset of the 2s kindling stimulus.